UNIVERSITY OF SOUTH BOHEMIA, FACULTY OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND BIOCHEMISTRY

Cytotoxicity screen of the acyclic nucleoside phosphonates against bloodstream stage of *Trypanosoma brucei* and validation of their putative target hypoxanthine/xanthine/guanine phosphoribosyltransferase

Martina Aistleitner
Biological Chemistry

Supervisor: Alena Zíková PhD
Institute of Parasitology, Biology Centre, ASCR, v.v.i.
Laboratory of Molecular Biology of Protists
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Annotation

The aim of this study was to test several compounds of acyclic nucleoside phosphonates (ANPs), which are thought to inhibit the purine salvage pathway enzyme hypoxanthine/xanthine/guanine phosphoribosyltransferase (HGXPRT) of the bloodstream stage of *Trypanosoma brucei*. Further we intended to elucidate if HGXPRT enzymes are essential for the survival of the bloodstream *T. brucei*.

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1. Introduction

1.1. Kinetoplastida, Trypanosomatida, genus Trypanosoma, Trypanosoma brucei

The phylum *Euglenozoa* is comprised of three groups, the *Euglenida*, the *Diplonemida* and the *Kinetoplastida* (Cavalier-Smith, 1993). The *Euglenida* are free living flagellates, mostly containing a green plastid, while the *Diplonemida* subdivide into few free living and mainly parasitic protists (Vickerman, 2000).The *Kinetoplastida* are a group of flagellates, categorized according to their life cycle and morphology into *Bodonina* and *Trypanosomatida* (Doležel *et al*., 2000, Simpson *et al*., 2002).While the *Bodonina* can be found in several various life cycles, free living as well as parasitic, the *Trypanosomatida* parasitize humans and all other classes of vertebrates, several invertebrates and also some plants. Invertebrates act as the only host for monoxenous trypanosomatids, but also as vectors for dixenous trypanosomatids, which are comprised of the genera *Trypanosoma*, *Leishmania* and *Phytomonas* (Vickerman, 1976). Trypanosomatida are the most studied subgroup of Kinetoplastida, because they include the medically and economically very important parasitic species of genus *Trypanosoma* and *Leishmania.*

T. brucei causes the sleeping sickness or wasting disease in Africa, while the South American parasite, *T. cruzi*, is responsible for Chagas disease and finally, the species of genus *Leishmania* are the causative agents of various forms of Leishmaniasis.

The species *Trypanosoma brucei* is comprised of three subspecies, *T.b. brucei*, *T.b.gambiense*, and *T.b.rhodesiense*, of which only *T.b. brucei* is not infective to humans due to its susceptibility to lysis by normal human serum (Vanhamme *et al*.; 2003). Because it shares many characteristics with the human pathogens *T.b. gambiense* and *T.b.rhodesiense*, and is morphologically indistinguishable, *T.b. brucei* is often used as a laboratory model.

1.2. Life cycle of Trypanosoma brucei

T. brucei passes through two life stages during its life cycle and undergoes drastic changes to accomodate the different environments. When the insect vector, its host, takes a blood meal, it ingests bloodstream trypomastigotes from the mammalian host.The bloodstream parasite then transforms into the procyclic stage in the insects midgut and multiplies by binary fission. As it moves to the salivary gland of its vector it goes through further transformations, until the parasite is injected back into the mammalian host during bloodmeal. In the bloodstream of the host it morphes into bloodstream trypomastigote and is carried to other sites where it mulitplies in various body fluids. Once the trypomastigote is in the blood, it can be detected and the infection diagnosised. In the advanced stage of infection the parasite can also cross the blood-brain barrier, giving rise to the neurological symptoms of the disease.

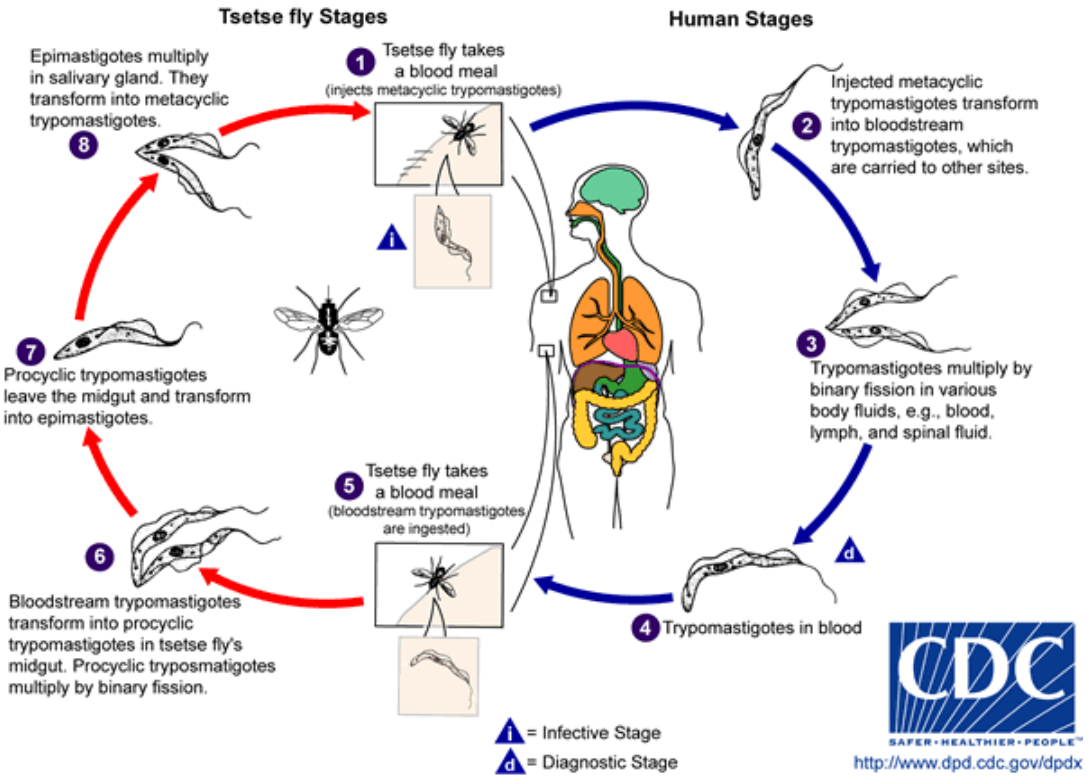

Figure 1: The life cycle of *Trypanosoma brucei*, with a short description of both life forms in its different stages: the procyclic form (Tsetse fly stages) and the bloodstream form (human stages). (www.dpd.cdc.gov/dpdx; March 3rd 2012).

1.3. Sleeping sickness, Human African Trypanosomiases (HAT)

Human African Trypanosomiasis (HAT), also known as the sleeping sickness, is a major cause of morbidity and mortality in 36 countries throughout sub-Saharan Africa threatening millions of people. The affected populations live primarily in remote areas with limited access to adequate health services, which complicates surveillance, therefore diagnosis and treatment of cases. Additionally displacement of populations, war and poverty lead to increased transmission.

T. brucei is the causative agent of sleeping sickness. The parasites are transmitted by the tsetse fly of the *Glossina* genus. They have in turn acquired their infection either from humans or animals harboring the pathogenic parasites. Though the disease is mostly transmitted through the bite of the tsetse fly, it can also be transmitted from mother to fetus, as well as by mechanical transmission through other blood sucking insects or from contaminated needles (WHO fact sheet N°259, Jan. 2012). Human populations which depend on agriculture, fishing, animal husbandry or hunting are most exposed to the tsetse fly and therefore to the HAT disease.

The majority of cases, 95% (WHO fact sheet N°259, Jan. 2012) are attributed to *T.b. gambiense* and which is found in West and Central Africa. This form causes chronic infection and can be dormant for several months or even years. When finally symptoms of the disease do emerge, the infected person is often in an advanced stage of the disease, where the parasite has already crossed the blood-brain barrier and the central nervous system is affected, which makes treatment difficult. *T.b. rhodesiense* is found in Eastern and Southern Africa and causes acute infection. The first symptoms become apparent several weeks or months after infection, and then the disease develops rapidly and invades the central nervous system. The species *T.b. brucei* is pathogenic to animals, causing a disease called nagana. Especially in domesticated animals it is a major obstacle of the economic development in the regions affected. Infected animals pose an additional threat to humans, because they can also host the human pathogen parasites *(T.b. gambiense and T.b. rhodosiense),* and act as parasite reservoir.

The HAT disease can stay dormant for several weeks to months or even years, and presents itself with the following progress and symptoms: in the first stage, called the haemolymphatic phase, the parasite multiplies in subcutaneous tissues, lymph and blood, and the patient experiences headaches, joint pains, itching and fever. In the second stage the trypanosomes cross the blood-brain barrier to infect the central nervous system. This is called the neurological phase. Generally the more typical and obvious signs and symptoms appear: confusion, changes of behaviour, sensory disturbances and poor coordination. Disturbance of the sleep cycle, which gives the disease its name, is an important feature of this second stage. Without treatment, sleeping disease is considered fatal.

There have been several epidemics in Africa over the last century. The epidemic had been controlled by mobile teams which organized screenings of millions of people at risk, and the disease had almost disappeared. After this success, surveillance was neglected, and HAT reappeared in several areas over the last 30 years. The efforts of the World Health Organisation (WHO), non-governmental organisations (NGOs), national control programmes and bilateral cooperation during the last two decades stopped and reversed the increasing numbers of new cases. Currently the number of actual cases is estimated at 30 000 infected persons (WHO fact sheet N°259, Jan. 2012).

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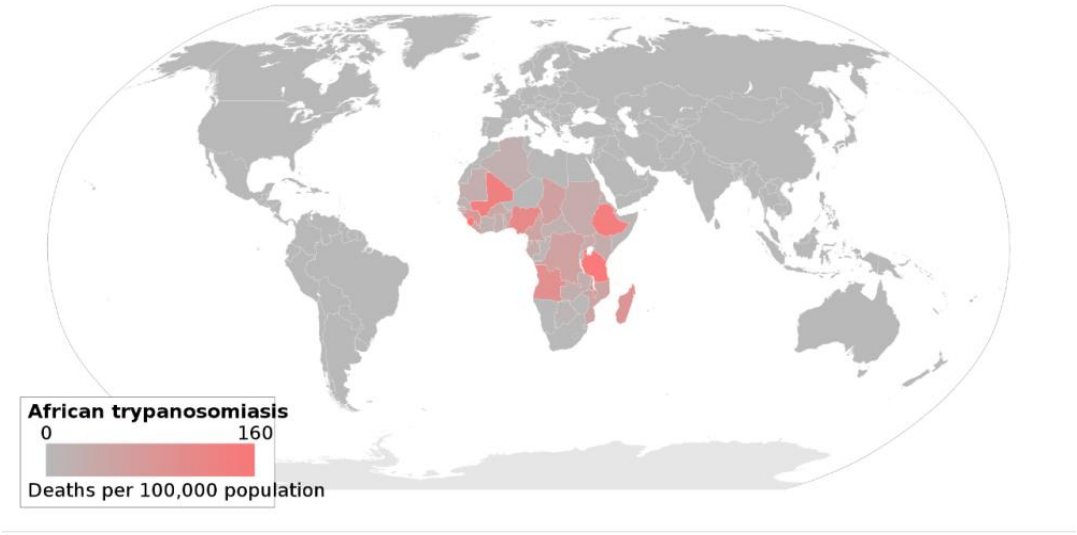


Figure 2: Deaths per 100 000 populations due to Human African Trypanosomiasis by country in 2002. (Richard Wheeler, Wikipedia; March 9th 2012; used with explicit permission)

For these patients, several treatment options are currently available. The type of treatment depends on the stage and the earlier the disease is diagnosed, the better the prospects of a cure. Generally the drugs used in the first stage are of lower toxicity and easier to administer. The success of treatment in the second stage depends on a drug that can cross the blood-brain barrier. Often these are toxic and difficult to administer.

The following four drugs are registered for the treatment and provided free of charge to endemic countries: (i) pentamidine is applied for the treatment of *T.b. gambiense* sleeping sickness in the first stage of the disease. It is generally well tolerated. (ii) suramin is used for treating sleeping sickness caused by *T.b.rhodesiense*, and often leads to allergic reactions and other undesirable effects and can also only be used to treat patients in the first stage of HAT. (iii) melarsoprol is effective against both forms of HAT in the second stage, but has many undesirable side effects, and can be fatal in rare cases. (iv) eflornithine treats second stage *T.b. gambiense*, but there is a strict and difficult regiment to be followed. A combination treatment of nifurtimox and eflornithine has been introduced in 2009, unfortunately it is not effective for *T.b.rhodesiense* (WHO fact sheet N°259, Jan. 2012). There are several problems with the treatments available. First of all, resistance against drugs effective for the first stage of HAT is increasing, and furthermore, the drugs used for the second stage are toxic and possibly fatal. (WHO fact sheet N°259, Jan. 2012). In total, the available drugs and treatments are unsatisfactory (Barrett Michael, 2006). Thus new drug targets have to be identified and the research on drug discovery speed up to meet the urgent need for new effective drugs.

The drug target discovery has been strongly facilitated in 2004 when the genome of *T. brucei* has been sequenced and analyzed. It is comprised of 11 megabase-sized chromosomes, which contain approximately 1700 genes specific for *T. brucei* (Berriman, *et al*., 2005) The analysis also showed why finding an effective vaccine has been proving difficult: large subtelomeric arrays contain and archive of 806 variant surface glycoprotein (VSG) genes. These are used by the parasite to evade the mammalian immune system. Most VSG genes are pseudogenes, which possibly are used to generate expressed mosaic genes by recombination, a process known as antigenic variation (Berriman, *et al*., 2005).

Apart from its two relatives *T. b. gambiense* and *T. b. rhodesiense*, *T. b. brucei* does not cause a sleeping sickness in humans and thanks to the striking similarity to its harmful relatives it has become a model system in the laboratory. *T. brucei* is an outstanding and interesting subject of research, not only because of its clinical importance but also because it possesses evolutionary unique molecular and biochemical properties.

In the process of drug discovery, metabolic pathways unique to the parasite are the main focus. One such pathway is the purine salvage pathway, because trypanosomatid parasites can not synthesize the essential purine molecules *de novo* as their mammalian host. Since the parasite is fully dependant on the purine salvage pathway, this pathway is considered a putative drug target.

1.4. Purine salvage pathway

Purines are of vital importance to all living organisms. Purine nucleotides function as precursors for nucleic acids (DNA and RNA), modulators of enzyme activities, and as constituents of certain co-enzymes (for instance ATP, NADH, coenzyme A). As purine nucleosides they can work as neurotransmitters by acting upon purinergic receptors, e.g. adenosine receptors.

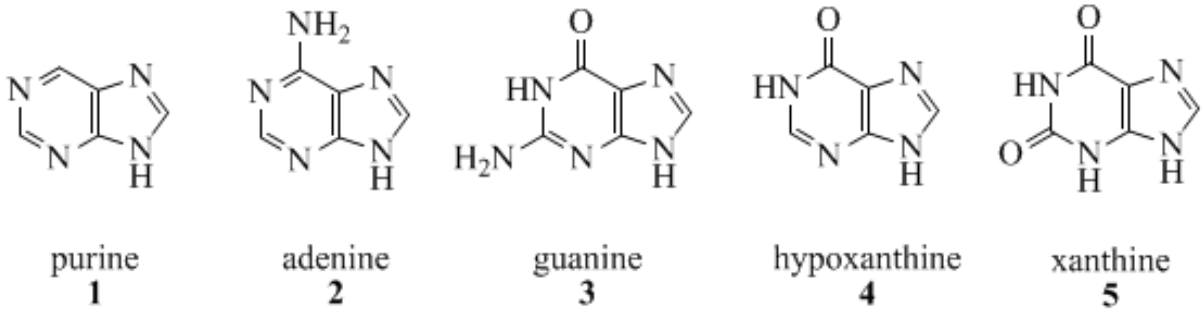


Figure 3: The different nucleobases, from which nucleosides and nucleotides are derived. (Wikipedia; 9.3.2012)

In general, purine nucleotides can be synthesized in two ways: by *de novo* biosynthesis, and/or by the several so-called 'salvage' pathways. Many mammalian cells possess the ability to use both pathways for their synthesis. However, in contrast to their host, nearly all parasites studied to date are obligatory purine auxotroph, incapable of *de novo* synthesis (el Kouni, 2003). The purine salvage pathway (PSP) is reutilization route by which the parasitic cell can meet its purine demands from endogenous and/or exogenous sources of preformed purine bases and nucleosides. Purine nucleobases such as hypoxanthine and xanthine are produced continuously in mammalian cells by the catabolism of purine nucleotides. There is therefore considerable opportunity for endoparasites of these cells to satisfy their purine requirement. Consequently, parasites have developed systems to transport, internalise and metabolise the required substrates. Usually the parasite can take up, interconvert and incorporate a variety of purine bases or nucleosides. The major purine source in human blood is a matter of controversy; however it seems that the most abundant purine molecules are adenosine and hypoxanthine (Slowiaczeck and Tattersall, 1982, Jabs *et al*., 1990). In *T. brucei* the nucleobase salvage efficiency is adenine, hypoxanthine, guanine and xanthine and the same order applies to nucleoside (Hammond, Gutteridge; 1984).

The salvage of host purines is initiated by their translocation across the parasite plasma membrane. Salvaged purines mainly enter the parasite in the form of either nucleobases or nucleosides via transporters, most of which are proton symporters. Investigation of the *T. brucei* genome has identified 12 distinct nucleoside transporters that mediate the uptake of nucleosides and/or nucleobases, and it remains unclear why the parasite requires so many of them (Ortiz, Sanchez, Quecke, Landfear; 2009). Some are specific to the bloodstream form (BF) or procyclic form (PF) of the parasite, and several show high substrate affinity, however it appears that the viability of the parasite is not entirely compromised with a single transporter knocked out (Berg M. *et al*., 2010). The fact that parasites simultaneously draw on multiple transport systems for uptake of both nucleobase and nucleoside purines renders blocking of these systems a complex and currently unfeasible approach in anti-parasitic therapy development. The transporters remain however of pharmacological importance as they provide the main entry route for several drugs to the parasitic cell (for example *T. brucei* drugs such as pentamidine and melarsoprol) (Berg M. *et al*., 2010).

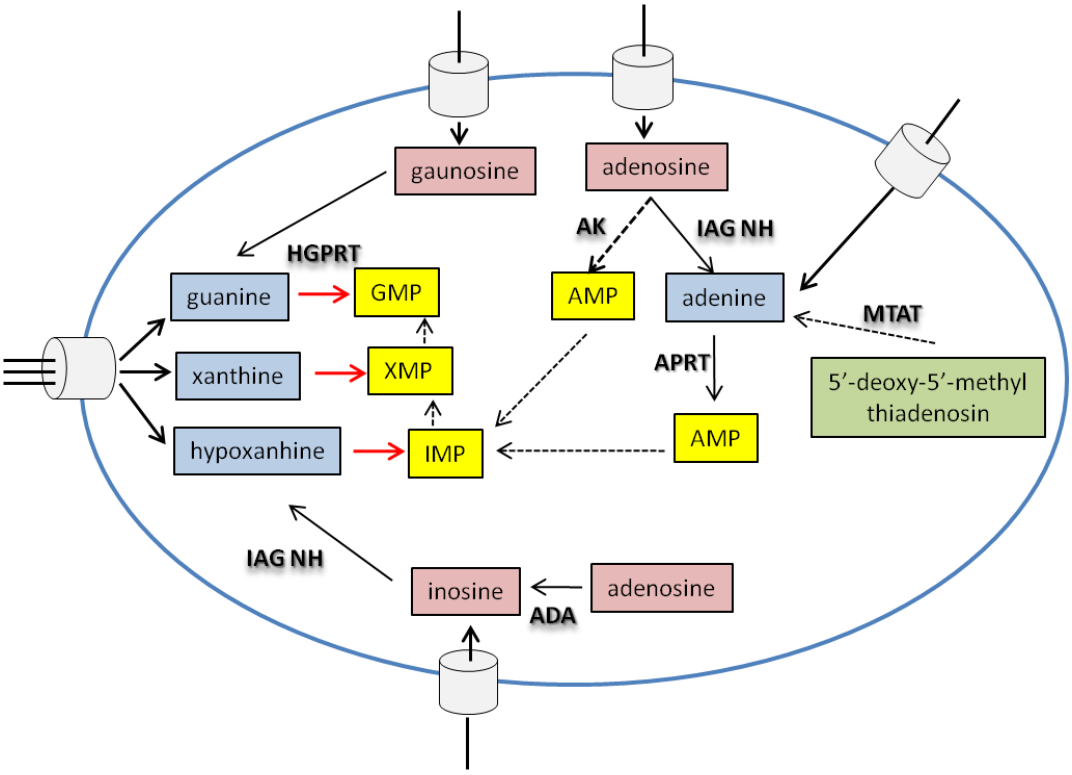


Figure 4: Scheme of purine salvage pathway in *Trypanosoma brucei*. HGXPRT – hypoxanthine/ guanine/ xanthine-phosphoribosyltransferase; AK – adenosine kinase; IAG NH – inosine/ adenine/ guanine - nucleoside hydrolase; APRT – adenosine phosphoribosytransferase; MTAP - Methylthioadenosine phosphorylase; ADA – adenosine deaminase; GMP, XMP, IMP, AMP – guanosine-, xanthine-, inosine-, adenosine monophosphate, respectively.

After an uptake of the purine molecules, the parasitic cell possesses a substantial number of enzymes involved in cleaving, assembling and interconverting these purines (Figure 4). First steps of purine salvage pathways involve catalytic enzymes, such as nucleoside hydrolase (NH), methylthiadenosine phosphorylase (MTAP) and phosphoribosyltransferases (PRTs) that catalyze reactions by transferring purines to or from ribose or deoxyribose centres. Both NH and MTAP are classified as cleaving enzymes using a nucleophile, which can be water or phosphate molecules, to cleave the glycosidic bond between the aglycon and the sugar ribosyl moiety. On the other hand, PRTs is a coupling enzyme catalyzing the reaction following the NH or MTAP activity: it transfers a phosphoribose unit onto the cleaved nucleobases, synthesizing purine nucleoside monophosphates (Figure 5) (Berg M. *et al*., 2010).

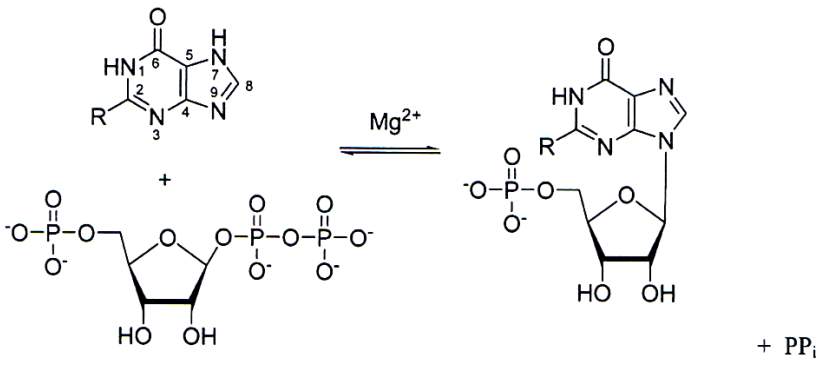


Figure 5: Reaction catalysed by phosphoribosyl transferase. For the purine base guanine R is $–NH_2$, for hypoxanthine –H and for xanthine R is OH (Keough *et al*., 2009).

Different types of PRTs have been found in many parasites and their specificity is variable within the genus (de Koning, Bridges, Burchmore; 2005). Two selective PRTs have been identified in the *T.brucei* genome. APRT has a distinct affinity to adenine and HGXPRT to 6-oxo purines. Even if the parasite's purine salvage pathway is very versatile, it is plausible to hypothesize that activity of HGXPRT is essential for the parasite and its inhibition would lead to cell death as the parasite would not be able to convert the salvaged 6-oxo purines into the necessary 6-oxo nucleosides. Mammalian cells also possess an HGPRT enzyme, but the selectivity of this enzyme differs from the parasitic HGXPRT since it does not have a binding affinity to xanthine. On the other hand, xanthine is a very good substrate for the parasite's HGXPRT enzyme. This different substrate selectivity might be important for future drug discovery. However even if the designed inhibitors would interfere with the mammalian HGPRT, it should not affect the mammalian cell since it has been shown that when humans inherit partial deficiencies of the activity of the corresponding enzyme HGPRT, they are able to lead normal lives, even if the activity of HGPRT is reduced to 3% of normal. The only side effect is urate overproduction, which can be easily treated (Keough *et al*., 2009). In this case the human cell utilizes only the *de novo* synthesis pathway to produce purine molecules.

1.5. Inhibition of HGXPRT by ANPs

The HGXPRT enzyme is considered to be a very promising drug target in *Plasmodium* parasites. Of the Plasmodium species, *Plasmodium falciparum* is the most lethal causing malaria. It remains a serious disease, resulting in 1-2 million fatalities each year, with approximately 48% of the world population living in areas of risk (Keough *et al*., 2009).

These parasites also lack the *de novo* pathway for purine nucleotide synthesis and they are dependent on salvaging purines from the host cell. Moreover, malaria parasites also lack some of the purine salvage enzymes that provide alternative pathways to purine nucleotides. Therefore the catalyst HGXPRT is essential for the survival of the parasite.

As it has been already mentioned, PRTs synthesize the purine nucleotide monophosphates required to produce nucleic acids. A class of purine nucleotide analogues, the acyclic nucleoside phosphonates (ANPs) have been investigated as potential inhibitors, as they are structurally very similar to the purine nucleoside monophosphates,

ANPs studied in this work are derivatives of purine bases, with three major differences in chemical properties to mononucleotides. First, the ester oxygen in the phosphate group is replaced by a carbon atom. This property ensures that enzymatic dephosphorylation or chemical hydrolysis of these analogues is excluded. Second, the position of the oxygen atom in the acyclic chain is shifted, compared to the position it would normally occupy in the ribose moiety. Third, the absence of a glycosidic bond in the structure of ANPs further increases their resistance to chemical and biological degradation. Another structural advantage is the flexibility of the acyclic chain, which enables the compounds to adopt a conformation suited for the interaction with the active site of the enzyme (Keough *et al*., 2009).

Importantly, ANPs have been shown to selectively inhibit PfHGXPRT compared to human HGPRT. The structure of both the purine base and the phosphonate moiety play significant roles in the affinity for the active site and in selectivity between the two enzymes (Keough *et al.,* 2009). The selected ANPs inhibited the growth of *P. falciparum* parasites grown *in vitro* at micromolar amounts and provided a basis for the design of more potent and selective ANP inhibitors as antimalarial drug leads. Since there are similarities between the *P.falciparum's* and *T. brucei's* purine salvage pathways, we decided to test the same set of ANPs against *T. brucei* BF stage. Furthermore using RNAi, we silenced an expression of TbHGXPRT genes in the bloodform (BF) of *T. brucei* in order to validate this enzyme as a putative drug target.

1.6. RNAi

RNAi stands for RNA interference, and is an effective tool for silencing the expression of a specific gene in *Trypanosoma brucei* (Ngo *et al*.; 1998). With this method, the function of a certain protein as well as its importance in an organism can be determined. Since the discovery of this manipulation technique, it has become a quick and widely used screening tool.

For *T. brucei* there are several vectors with different mechanisms to produce double stranded RNA (dsRNA) which triggers the RNAi. The head-to-head T7 promoters can be employed to express dsRNA or one T7 promoter drives an expression of a stem loop RNA which incorporates a 'stuffer' region between 2 inverted repeats of the gene. Then an enzyme, called Dicer, binds to and cuts the RNA into pieces of approximately 20 nucleotides, termed small interfering RNA (siRNA). In turn, siRNA interact with the RNA-induced silencing complex (RISC), which binds single strand siRNA to complementary sequences of single-stranded mRNA. Once RISC is bound to mRNA, it silences the expression of the gene by degrading its mRNA. All the necessary enzymes and other building blocks for RNAi are present in *T. brucei*, which makes it a splendid technique to examine genes for their function and significance.

2. AIMS

1. To establish a cytotoxicity assay to measure viability of *T. brucei* cells
2. To test a small library of acyclic nucleoside phosphonates
3. To validate HGXPRT enzymes as putative drug targets in *T. brucei*

3. Methods

3.1. AlamarBlue® assay

The AlamarBlue® cell viability reagent works as a health indicator for cells of different kinds. Living cells maintain a reducing environment inside the cytosol of the cell. The active ingredient of the alamarBlue® reagent is resazurin, which is a blue, virtually non-fluorescent, cell permeable indicator dye. When it enters the cells, resazurin is reduced to resorufin, which is red and highly fluorescent. It is basically a detector of metabolic activity (Lancaster, MV and Fields, RD, 1996). Using this property, the assay detects the relative cytotoxicity of applied compounds to the cell, as viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding the cells (Figure 6).

A. B.

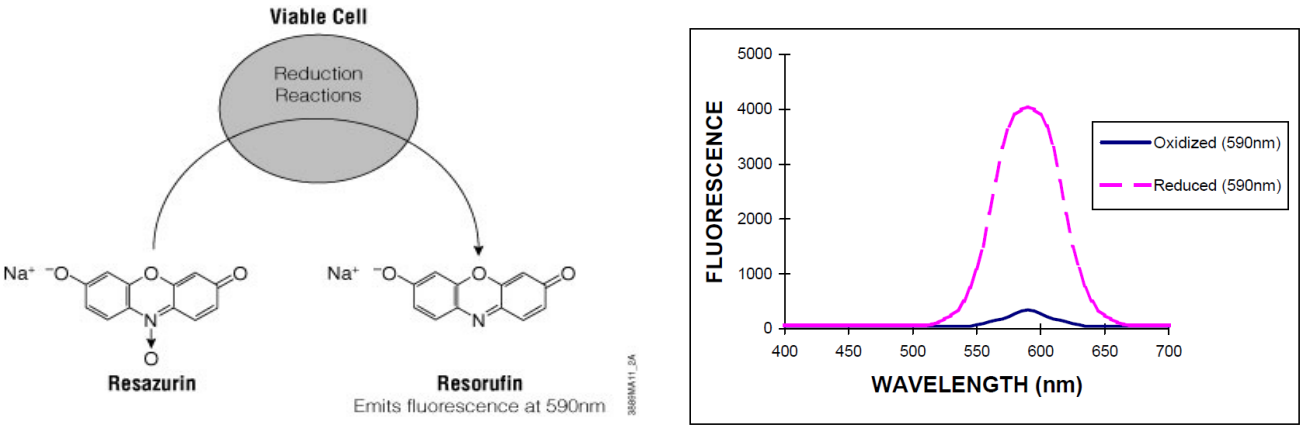


Figure 6: Scheme of resazurin reaction.

A. Reduction of resazurin to resorufin. B. Resazurin/resorufin reagent fluorescence emission spectra (TREK Diagnostic Systems for Invitrogen Ltd, 2008)

For the pilot experiments determining the linearity and sensitivity of the assay resazurin solution was prepared at a concentration 125µg/ml and filter sterilized.

BF cells ($1x10^5$ cells/ml and $1x10^6$ cells/ml) were distributed into the 96-well plate to create a range of concentration from $1x10^3$ cells/well to $1x10^5$ cells/well (Table 1a).

Table 1a. Distribution of cells and media

Well	1st	2nd	3rd	4th	5-6th	12th
Cells [$1x10^5$ cells/ml]	0	10µl	20µl	30µl	etc.	100µl
Concentration [cells/well]	0	$1x10^3$	$2x10^3$	$3x10^3$	etc.	$1x10^4$
HMI-9 Media [µl]	100	90µl	80µl	70ul	etc.	0µl

Table 1b.

Well	1st	2nd	3rd	4th	5-6th	12th
Cells [$1x10^6$ cells/ml]	0	10µl	20µl	30µl	etc.	100µl
Concentration [cells/well]	0	$1x10^4$	$2x10^4$	$3x10^4$	etc.	$1x10^5$
HMI-9 Media [µl]	100	90µl	80µl	70ul	etc.	0µl

To each well 10µl resazurin solution were added, and the plate was kept at 37°C. The fluorescence was measured 2, 5, and 24 hours after resazurin addition.

For drug treatment experiments the 96-well plate was filled with 50µl of HMI-9 media. 10µl of the studied drug solution (pentamidine at 100µM, ANPs at 10mM) was pipetted into the last well, and mixed shortly.

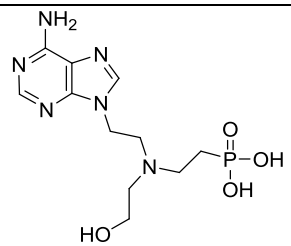
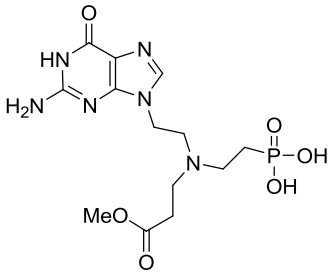
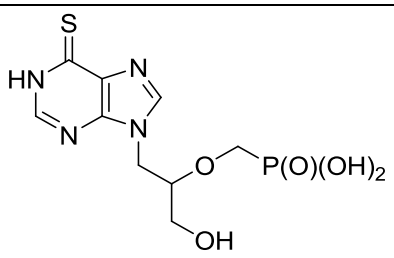
Out of the last well, 10µl solution was taken and added into the next well (generating dilution by 6). The dilution was continued until the last well. By this method the drug solution was diluted along the row giving several different concentrations. Further 50µl of $8x10^5$ cells/ml were added into each well (diluting the compound again by dilution of 2), resulting in a final cell concentration at $4e^4$ cells/well. The plates were left for 24 hours at 37°C, then 10µl of resazurin solution was added and the fluorescence was read after additional 4 hours of incubation at 37°C.

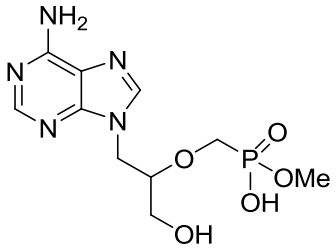
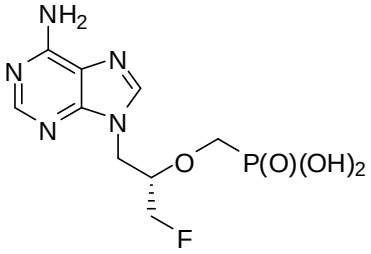
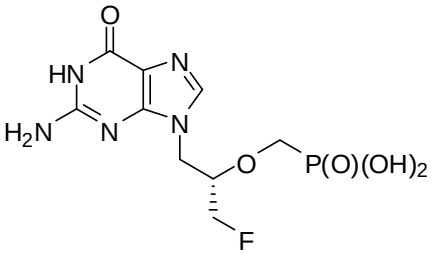
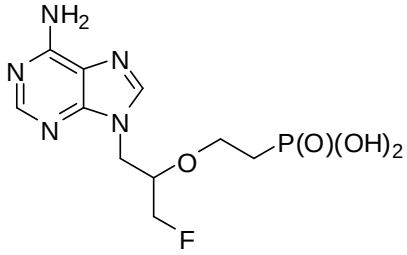
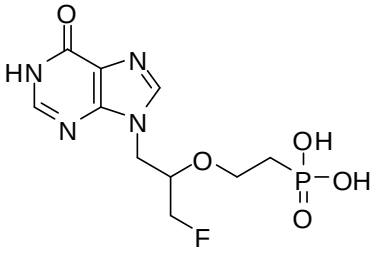
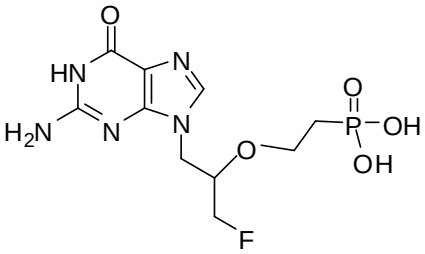
The resulting fluorescence was read on a microplate reader (TECAN) using fluorescence excitation wavelength of 540-570 nm and reading emission at 580-610 nm. The results were analyzed by plotting the fluorescence intensity versus compound concentration using Graph Pad Prism software.

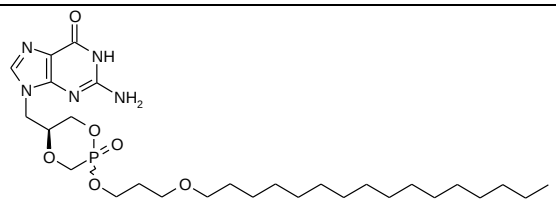
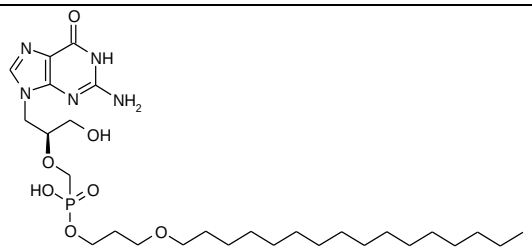
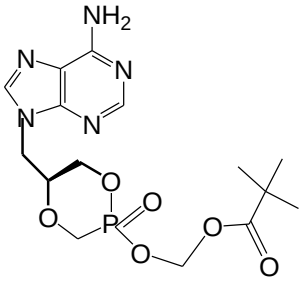
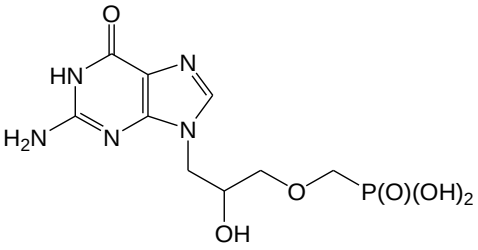
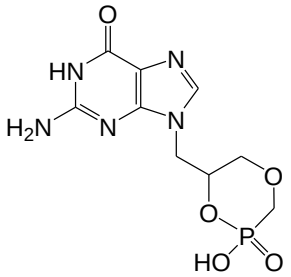
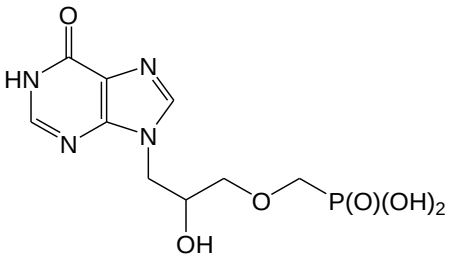
3.2. Selected acyclic nucleoside phosphonates

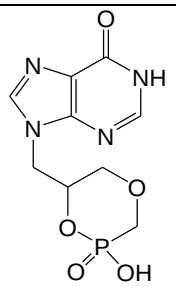
The compounds were kindly provided by Dr. Dana Hocková, Institute of Organic Chemistry and Biochemistry, AVCR.

Table 2: Library of tested ANPs

Compound	Structure	MW
DA-XI-48		330
DA-XI-56		388
H-2526		320

H-2578		317
OB-374		305.2
OB-214		321.2
OB-467		319.23
OB-464		320.21
OB-469		235.23

ESTER PRODRUGS		
TT-280510 (S)-cHPMPG-HDP		583,71
TT-200510 (S)-HPMPG-HDP		601,73
MK-717 (S)-cHPMPA-POM ester		399.34
FREE PHOSPHONIC ACIDS		
MK-455 "iso"-HPMPG, racemate		319.21
MK-697 "iso"-cHPMPG, racemate		301.20
MK-520 "iso"-HPMPHx, racemate		304.20

MK-747 "iso"-cHPMPHx, racemate		286.18

3.3. Growing BFT. brucei

The BF of *T. brucei brucei* (BF 427) cells were continuously cultivated throughout the duration of the experiments. This strain of *T. brucei* is generally used for studies because it poses no harm to humans. The bloodstream form cells were kept in a HMI-9 media with 10% foetal bovine serum (Hirumi *at al.*, 1997). This medium contains all the necessary nutrients and purines (hypoxanthine) for the cultured cells. Since the bloodstream cells maximum concentration in media is $2x10^6$cells/ml and the population doubles approximately every 7 hours, the cultures were diluted when necessary to keep the cells in a viable concentration. For RNAi experiments the genetically improved BF 427 cell line (single marker, SM) was used (Wirtz *et al*., 1999). This SM cell line expresses T7 RNA polymerase and tetracycline repressor allowing inducible expression of dsRNA from p2T7-177 vector (Wickstead *et al.*, 2002). Drugs to maintain selection for transgenes were included when relevant: G418 (2.5 μg/ml), phleomycin (2.5μg/ml).

3.4. Cloning of HGXPRT genes into RNAi vector p2T7-177

3.4.1. PCR

To amplify the desired genes, PCR was run with specifically designed primers. This technique is based on a polymerase enzyme which synthesizes a complementary strand to a given DNA strand using primer sequences flanking the target sequence. The reaction mixture goes through several temperatures; first it is heated to separate the double-stranded DNA into single stranded DNA, and then cooled to allow the primers to bind to their complementary sequences and the polymerase enzyme to synthesize the new strands. When the temperature cycle is repeated, the target DNA is multiplied exponentially (Figure 7.) (www.ucl.ac.uk; April 28th 2012).

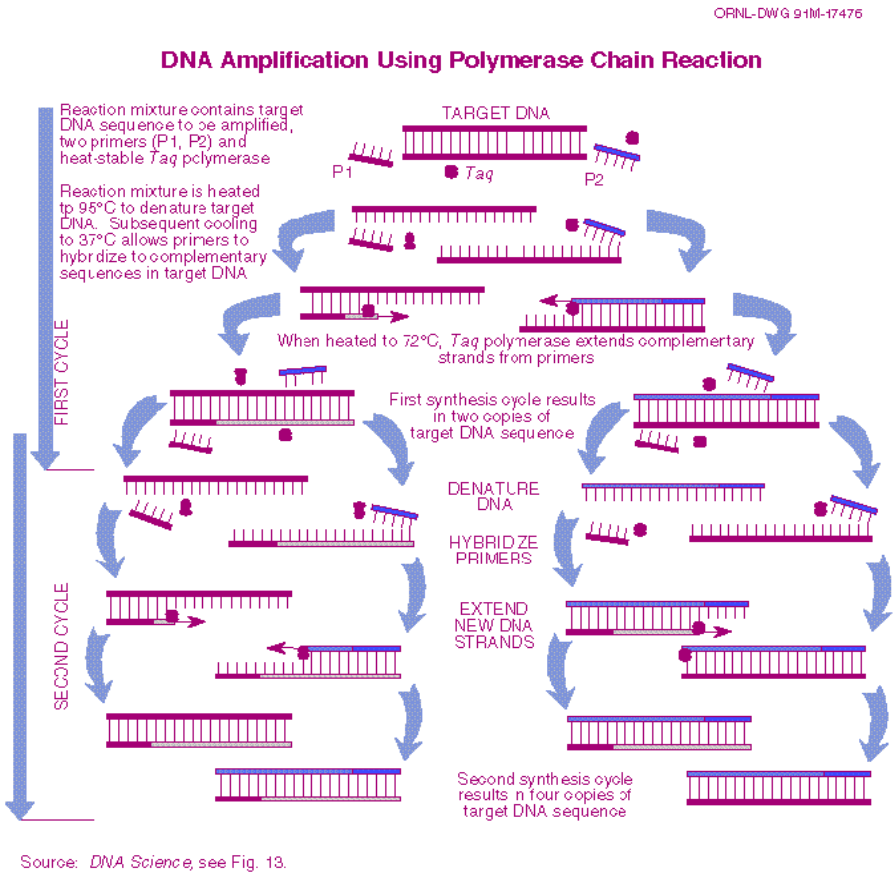


Figure 7: PCR cycle (www.ucl.ac.uk; April 28th 2012)

There are three genes encoding the putative HGXPRT enzyme in *T. brucei* the genome database (www.genedb.org).

Gene ID: Tb927.10.1390

Gene size: 703 bp

Protein size: 26kDa

Protein:

MHSGHPLKPN FVGRDADGNV TVDGRSYPMA ESVVATESTI HRSMKEMAQT LANAYKTLKH
RDTHNKGNSA LAPITDENPL IIISVLKGSY IFTADMVRYL GDCGLPNVVD FIRITSYRGT
TKSSGTVQVL DNLRFTELTG KHVLIMEDIA DTGRTMKLLV EKIRREYRPA SLKVCVLVDK
PGGRVVDFKP EFVCLTAPTR YVVGYGFEVN DRYRNYRHVF VLKPEYAKRY PSKL

Gene:

ATGCACTCGGGCCATCCTCTCAAACCGAACTTTGTTGGCAGGGATGCTGACGGAAATGTTACGGTT
GATGGCAGATCATATCCCATGGCGGAAAGTGTTGTCGCCACGGAGAGCACAATTCACCGAAGCAT
GAAGGAAATGGCACAGACGCTTGCCAACGCTTACAAGACACTTAAGCACCGCGATACCCACAATA
AGGGTAATAGCGCATTGGCCCCAATCACCGATGAAAATCCACTAATTATTATTTCTGTCCTGAAGG
GCAGTTATATTTTCACGGCAGACATGGTTCGTTACCTTGGAGACTGTGGGCTACCTAACGTTGTGGA
CTTTATACGCATTACTTCATACAGGGGAACCACGAAAAGCAGTGGCACCGTGCAGGTGCTGGATAA
CCTGCGCTTCACTGAATTAACGGGGAAACATGTACTTATTATGGAAGACATTGCCGACACGGGGCG
TACAATGAAGCTTTTGGTGGAGAAGATCCGCCGGGAGTACCGACCTGCCAGTTTGAAGGTGTGCGT
ACTTGTGGACAAGCCCGGTGGTCGCGTTGTGGACTTTAAGCCCGAATTTGTTTGCCTGACTGCACCA
ACTAGATACGTGGTTGGTTACGGCTTCGAGGTGAATGACAGGTACAGGAACTACCGTCACGTCTTT
GTTTTAAAACCGGAGTATGCGAAACGGTACCCGAGCAAATTGTAA

Gene ID: Tb927.10.1400 and Tb927.10.1470

Gene size: 630bp

Protein size: 23kDa

Protein:

>Tb927.10.1400

MEPACKYDFA TSVLFTEAEL HTRMRGVAQR IADDYSNCNL KPLENPLVIV SVLKGSFVFT
ADMVRILGDF GVPTRVEFLR ASSYGHDTKS CGRVDVKADG LCDIRGKHVL VLEDILDTAL
TLREVVDSLK KSEPASIKTL AIDKPGGRK IPFTAEYVVA DVPNVFVVGY GLDYDQSYRE
VRDVVILKPS VYETWGKELE RRKAAGEAKR

>Tb927.10.1470

MEPACKYDFA TSVLFTEAEL HTRMRGVAQR IADDYSNCNL KPLENPLVVV SVLRGSFVFT
ADMVRILGDF GVPTRVEFLR ASSYGHDTKS CGRVDVKADG LCDIRGKHVL VLEDILDTAL
TLREVVDSLK KSEPASIKTL AIDKPGGRK IPFTAEYVVA DVPNVFVVGY GLDYDQSYRE
VRDVVILKPS VYETWGKELE RRKAAVKAKL

Gene:

>Tb927.10.1400

ATGGAACCAGCTTGCAAATACGACTTCGCAACGAGTGTCCTCTTTACAGAGGCAGAACTACACACT
CGCATGCGCGGTGTGGCGCAGCGTATTGCCGATGACTACAGCAACTGCAATTTGAAGCCACTTGAA
AATCCTCTGGTAATTGTGTCTGTATTGAAGGGCAGCTTCGTCTTCACTGCTGACATGGTTCGCATTC
TTGGTGATTTTGGCGTCCCCACACGTGTGGAATTCCTACGGGCCTCGTCATATGGTCACGATACTAA
AAGTTGTGGTCGAGTTGACGTGAAGGCTGACGGTCTTTGTGACATCCGCGGCAAACATGTCCTTGT
TTTGGAGGATATACTTGATACTGCGCTGACGTTGAGGGAAGTGGTGGACAGCTTGAAAAAGAGCG
AACCCGCGAGCATTAAAACCCTCGTGGCTATCGACAAACCCGGTGGACGTAAAATACCTTTCACTG
CGGAATACGTTGTGGCGGATGTTCCCAATGTGTTCGTGGTTGGCTACGGGTTGGATTACGACCAAT
CATACCGTGAGGTGCGTGATGTTGTAATCCTAAAACCGAGCGTGTATGAAACATGGGGAAAGGAA
CTTGAGCGGAGGAAGGCCGCTGGAGAAGCCAAGCGGTAA

For PCR the following primers were used:

Tb1390 FW (XhoI): CTCGAGATGCACTCGGGCCATC

Tb1390 Rev (HindIII) : AAGCTTCATTGTACGCC

Tb1400 FW (XhoI): CTCGAG ACGACTTCGCAA

Tb1400 Rev (HindIII) : AAGCTT GTCGATAGCCACGAGG

In the following runs, the quantities of DNA were amplified via PCR. Taq polymerase was used because it inserts overlapping adenosine tails to the PCR products, which proved helpful when the gene was inserted into the plasmid later.

Reagents:

dNTPs (10mM each)	1µl
10x blue buffer	5µl
Taq-purple	0.5µl
Template gDNA (100ng)	1µl
primers: FW and Rev (10µM)	1+1µl
MilliQ H_2O	total: 50µl

Temperature Cycle:

Step 1: 95°C … 5 min

Step 2: 94°C … 0.5 min

Step 3: 55°C … 0.5 min

Step 4: 72°C … 0.5 min - 30 repeats

Step 5: 72°C .....7 min

Step 6: hold at 4°C

Step 2-4 were repeated 30 times.

3.4.2. DNA Gel Electrophoresis

The DNA fragment from PCR was fractioned and visualized using gel electrophoresis. For a 0.75% agarose gel, the agarose was mixed into TAE buffer (2M Tris-base, 5,71 % acetic acid, 5mM EDTA ph 8) and heated for 100 seconds to melt the agarose. The solution was cooled before a maximum amount of 5µL ethidium bromide (5 mg/ml) was added. The gel was poured into the apparatus, the appropriately sized comb added and the gel cooled to polymerize for approximately 15 minutes. After the comb was taken out, the 1 Kb Plus DNA ladder (Figure 9) was applied to the first well, while samples including a loading dye was applied to the subsequent wells. Then the gel was run first with 70V, then 90V for approximately 45 minutes, possibly longer or shorter, depending on the size of the samples.

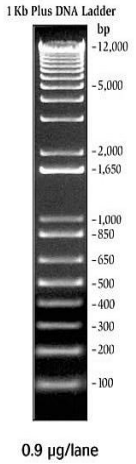


Figure 8: DNA ladder (Invitrogen)

The QIAGEN gel extraction kit was used to purify DNA from the gel.

3.4.3. Ligation of DNA inserts into the appropriate plasmids

The DNA purified from the gel was prepared to be inserted into a vector, using T4 DNA ligase. During the ligation reactions, hydrogen bonds form between the overhangs on the DNA fragments, and then the ligase repairs the phosphate backbone, creating a stable circular plasmid (ocw.mit.edu/index.htm, April 28th 2012).

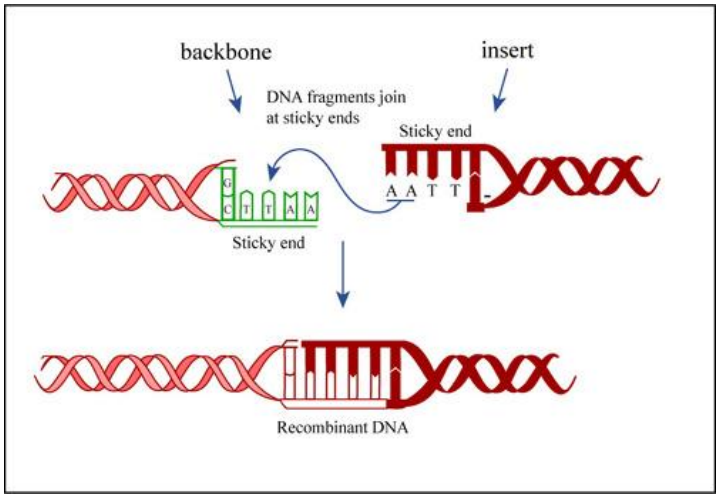


Figure 9:Ligation reaction (ocw.mit.edu/index.htm, April 28th 2012)

The purified PCR product was ligated into pGEM-T easy vector (Promega) using A/T overhands (Figure 10).

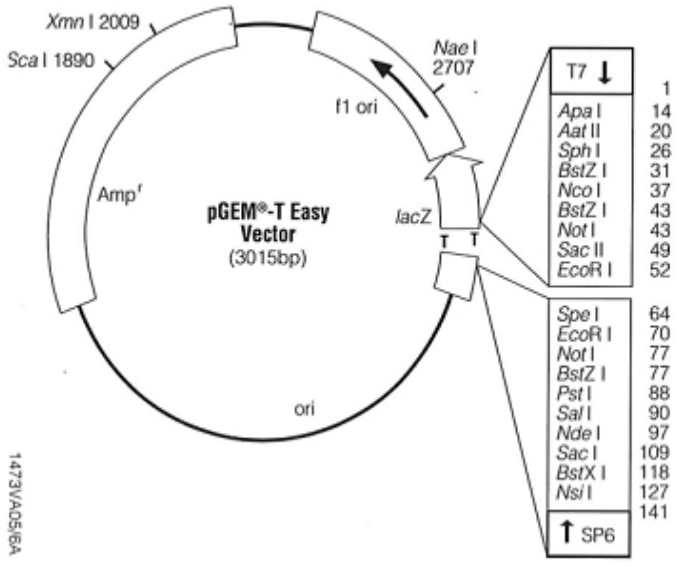


Figure 10: pGEM-T Easy Vector (Promega)

The Tb1390 and Tb1400 inserts were ligated into p2T7-177 vector (Wickstead *et al.*, 2002) (Figure 11).

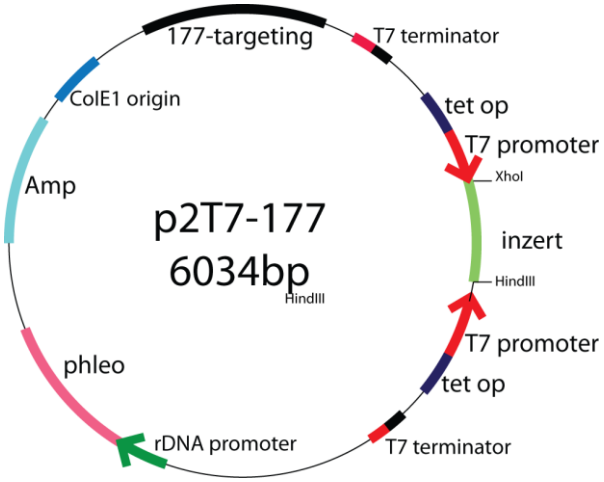


Figure 11: p2T7-177 vector (Wickstead *et al.*, 2002)

The molarity of the vector and of each insert was calculated using the following formula:
molarity [µM] = DNA concentration [µg μL^{-1}] / 0.325g mol^{-1} * length of DNA [bp]
pGEM-T easy: 3015 bp, 50 ng μL^{-1} → 50 nM
The vector and insert were mixed in a 1:3 molarity ratio.

Ligation mix with pGEM-T easy:

pGEM-T easy (25nM)	0.5 µL
insert (75nM)	3 µL
2x Ligation Buffer	5 µL
MilliQ H_2O	1 µL
T4 Ligase (Promega)	0.5 µL

The reaction was held at room temperature for 2 hours.

Ligation mix with p2T7-177:

p2T7-177 XhoI/Hind III	0.06µM - 1.5 µl
Insert XhoI/HindIII	Tb1390: 0.04µM – 6µl Tb1400: 0.06µM – 4.5µl
10x ligation buffer	1.5 µl
T4 DNA ligase	1µl
MiliQ H_2O	total: 10µl

The reaction was held at 12°C overnight.

3.4.4. Transformation into *Escherichia coli* cells

The ligation mixture containing circular plasmids was used to transform *E. coli* cells in which the plasmids containing the DNA of interest are multiplied and produced in a large quantity. To detect if a vector has incorporated the desired DNA fragment, the gene lacZ, which codes for β-galactosidase is utilized as marker gen. The usually colourless indicator X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) is cleaved by the enzyme to yield an insoluble blue compound similar to indigo. If a DNA fragment is inserted, the expression of the gene is disrupted, and it can't metabolise X-Gal, leading to white colonies, while bacteria without the insertion grow blue colonies. To ensure the function of the gene lacZ, a compound similar to a lactose metabolite that triggers the transcription of lacZ called Isopropyl-D-thio-galactoside (IPTG) is used as an inducer. IPTG binds to the repressor of the whole gene complex and inactivates it. Because the IPTG sulphur atom creates a non-hydrolysable chemical bond, it can not be metabolized by *E.coli*, preventing the cell from degrading it, keeping the IPTG concentration at a constant (http://www.agscientific.com/, April 2012).

To initiate the process, first the competent cells, in this case XL1 blue, were gently thawed and placed on ice for 10-20 minutes. XL1 blue cells contain the selection mechanism to easily determine if the transformation was successful via white/blue selection.
The ligation reaction (2-3 µl) was added to 50µl of the competent cells and the cells were kept on ice for 10-20 minutes, while occasionally mixing. The cells were then heat shocked at 42°C for 45 seconds in a water bath or dry block, while paying close attention to time and temperature, as this step is critical for the procedure. The cells were cooled down for 2 minutes. 250µL of sterile SOC medium (2% bakto-trypton; 0.5% Yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10mM $MgSO_4$; 20mM glucose) was added to a final volume of 300µL. The cells were incubated at 37°C in a shaking incubator for 45 minutes up to 1.5 hours. 100µL of the transformed cells were spread onto a plate containing ampicillin (final concentration at 100µg/ml). In the case of blue-white selection X-gal (30µl of 20mg/ml) and IPTG (100µl of 100mM) was spread on a plate. The plates were incubated at 37°C overnight.

3.4.5. Plasmid DNA isolation - Miniprep

A test tube with 3ml LB media to which ampicillin (final at 100µg/ml) had been added was inoculated with a single culture from the overnight transformation plate. This tube was incubated overnight in a shaking incubator at 37°C. The bacteria cells were then spun down at 5000rpm at 4°C in a 1.5ml test tube, and the plasmid DNA was isolated using QIAGEN Spin Miniprep kit following the manufacture's protocol.

3.4.6. Restriction digest of the plasmid DNA

Approximately 400ng of plasmid DNA were digested with sufficient amounts of the enzymes XhoI and HindIII (usually 10U to 1µL DNA, as 1U cut 1µg DNA per hour). Because enzymes were stored in 50% glycerol, caution had to be taken that the total glycerol content of the reaction did not exceed 4-5%. The restriction reaction containing appropriate buffer, DNA and enzymes was mixed well and incubated at 37°C in a heat block for 1.5 hours. The digested plasmid DNA was run on a 1% agarose gel to verify successful digestion.

3.4.7. Plasmid DNA isolation - Midiprep

To increase the amount of DNA for transfection, cultures with successful transformations were picked off the plate and inoculated to 100ml of LB media (ampicillin at 100µg/ml) to start growing the cultures. The plasmid DNA was extracted according to the QIAGEN Plasmid Midikit protocol.

3.5. Transfection of the bloodstream stage of T. brucei

3.5.1. Linearization of the p2T7-177/Tb1390 and p2T7-177/Tb1400 plasmids

To introduce the RNAi construct into the genome of *T. brucei* the p2T7-177 vector was linearized using the restriction enzyme NotI. The reaction mixture was composed of:

DNA	30µg (1390: 8µl, 1400: 28µl)
NOT1 buffer	5µl
NOT1	1µl
100x BSA	0.5µl
MilliQ H_2O	total : 50µl

The reaction was incubated at 37°C for approximately 7 hours, then precipitated by adding 2.5 times the volume of 96% ethanol and one tenth of volume of 3M sodium acetate with pH 5.2 . After at least 30 minutes at -80°C, the solution was centrifuged and the ethanol was pipetted off. The pellet was washed with cold 70% ethanol and subsequently centrifuged for 10 minutes. The ethanol was again pipetted off and the pellet was air-dried in the sterile flow box. The linearized DNA was resuspended in 20µL sterile H_2O.

3.5.2. Transfection of bloodstream *T. brucei* using the AMAXA Nucleofactor apparatus

The bloodstream cells ($3x10^7$ cells) were harvested at the concentration of $0.8x10^6$ cells per ml at 1300*g for 10 minutes in a swing bucket rotor. The media were poured off and the cell pellet was washed once with 20ml sterile PBS-G (5.6 mM Na_2HPO_4 x $12H_2O$, 3.6 mM $NaH_2PO_4x2H_2O$, 0.145 M NaCl, 3.3 mM glucose). The cell pellet was then resuspended in 100µL of AMAXA Human T-cell solution and transferred into the electroporation cuvette. The linearized plasmid DNA (10 – 12 µg) was then added. The cuvette was placed into the electroporator, and the appropriate program was turned on to electroporate the cells. In the meantime, 90ml of HMI-9 medium with the appropriate drugs (G-418, final concentration at 2.5µg/ml, HMI-9/G) was distributed among three 50ml sterile tubes in the following fashion: the first tube contained 30ml, while the other two were filled with 27ml.

The entire cell-DNA transfectants was transferred to the first tube and inverted several times to mix it well. The cell density in the first tube was now equal to 10^7cells/ml. The cell solution was further diluted 10 times into the second tube (final cell density 10^6cells/ml) and again diluted 10 times into the third tube (final cell density $1x10^5$ cells per ml). Aliquotes of 1ml were distributed into one 24 well plates and incubated at 37°C.

After 16 hours, 75ml of HMI-9/G media was prepared containing the necessary antibiotic phleomycin (at 2x concentration, 5µg/ml). 1ml aliquot of this media was added to each well in the plate, except for well A1, to which only the media without the drug was added. Cells resistant to phleomycin and thus containing the linearized plasmid in their genomes were observed at day 5 or 6 after transfection.

4. Results

4.1. Optimization of the assay

The effect of the number of cells per well and incubation time with resazurin solution was determined for BF cells. Cells were brought to appropriate concentration in media, 10µL of resazurin solution was added, and after 2, 5 and 24 hours incubation at 37°C, the absorption was measured.

To establish the linearity and sensitivity of the resazurin solution, several different concentrations of BF cells were incubated with 10µL of resazurin. Additionally the incubation time varied to determine the optimum for a significant response.

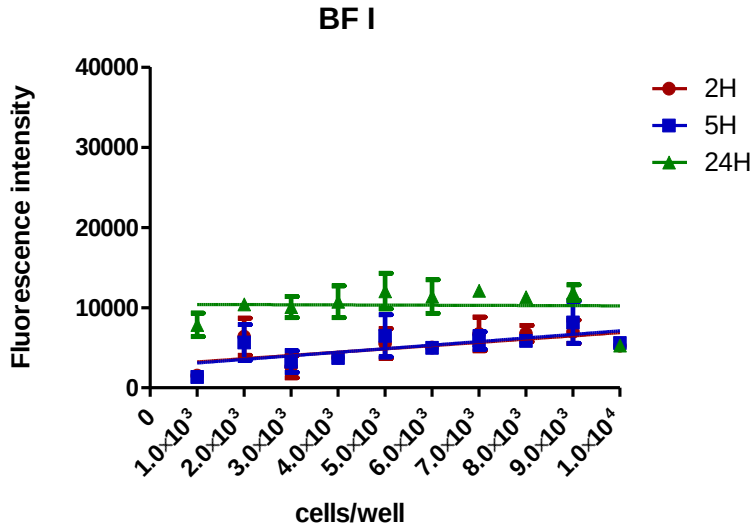


Figure 12. Standard curve of *T. brucei* BF cells. Cell concentration per well is depicted in the graph. The resazurin incubation time is illustrated by different colors. 2 hours – red, 5 hours – blue, 24 hours – green. Each point is the mean of two individual experiments.

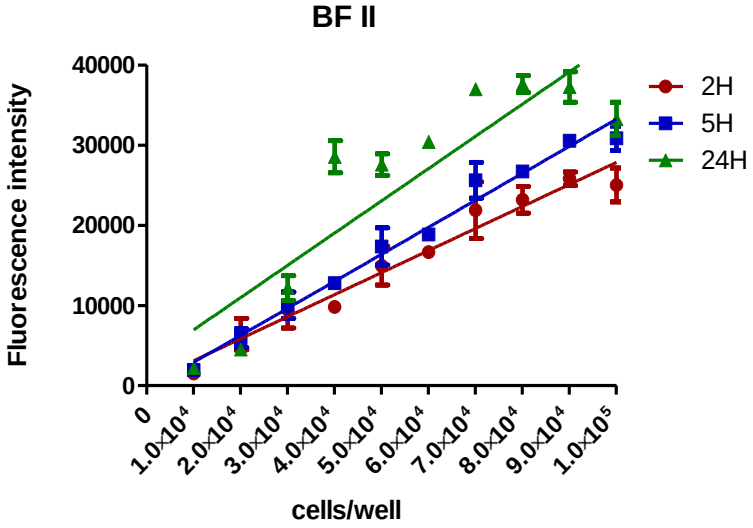


Figure 13. Standard curve of *T. brucei* BF cells. Cell concentration per well is depicted in the graph. The resazurin incubation time is illustrated by different colors. 2 hours – red, 5 hours – blue, 24 hours – green. Each point is the mean of two individual experiments.

When the dilution series of $1x10^3$ cells/well up to $1x10^4$ cells/well was used, the fluorescence intensity resafurin was too low to obtain satisfactory response (Figure 12). However the resazurin reductions was linearly associated with the number of parasites in the range $1x10^4$ to $1x10^5$ cells/well and incubation time 2 and 5 hours (r= 0.9166 and r=0.9658 respectively) (Fig. 13).

To demonstrate the applicability of our colorimetric assay against bloodstream stage of *T. brucei*, we evaluated the EC50 value of a common drug, pentamidine, used to treat sleeping sickness. The logarithmic growth phase culture was diluted down to $8x10^5$ cell/ml and 50µl of cells were distributed in 96-well plate. The cells were then diluted by 50µl of media with decreasing concentration of a drug. The cells were incubated for 24 hours, then 10µl of resazurin solution was added into each well and the cells were left in an incubator for additional 4 hours.

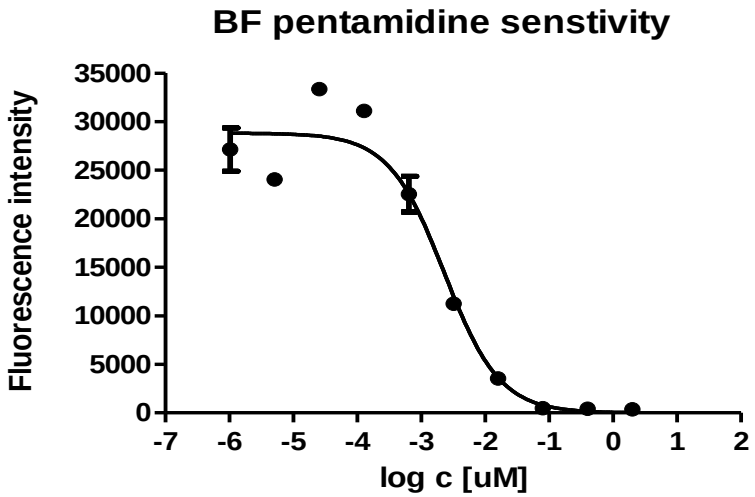


Figure 14: Fluorescence intensity vs. log of pentamidine concentration to determine the sensitivity of the resazurin measurements.

The calculated EC50 value of pentamidine treatment is approximately 2nM (Figure 14), which is in agreement with the published data (Raz *et al.*, 1997).

This pilot experiment verified applicability and sensitivity of our assay.

4.2. Cytotoxicity screen of selected ANPs against BF T. brucei

16 selected ANPs were tested under same conditions as described in the last chapter. All these compounds were dissolved in water and heated to 70°C to increase the solubility. Out of 16 compounds, 2 compounds showed a noteworthy effect on BF cells. The determined EC50 values for TT-200510 and MK-717 were 3µM and 82µM, respectively.

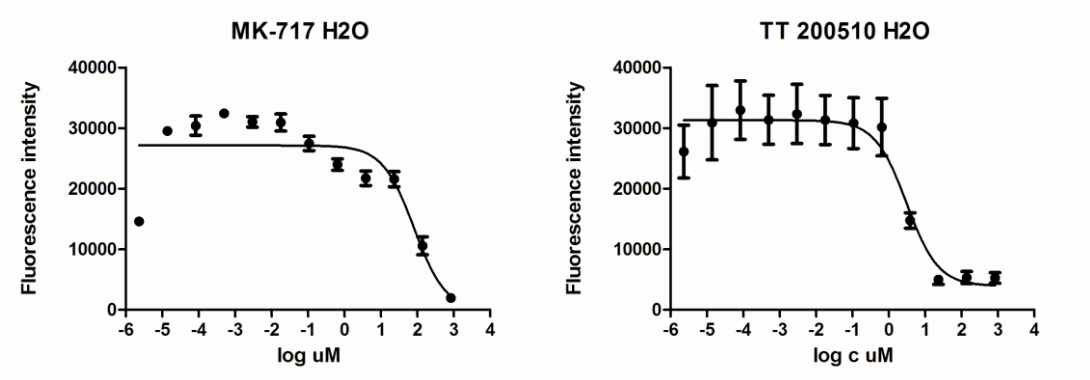


Figure 15: Fluorescence vs. log of concentration of respective ANPs;

Since both compounds were only partly soluble in water, the solubility was improved by dissolving both compounds in DMSO. Importantly, the EC50 value decreased significantly for MK 717 compound (now being at 6 μM). On the other hand, EC50 value for TT 200510 decreased only slightly (Figure 16, Table 3).

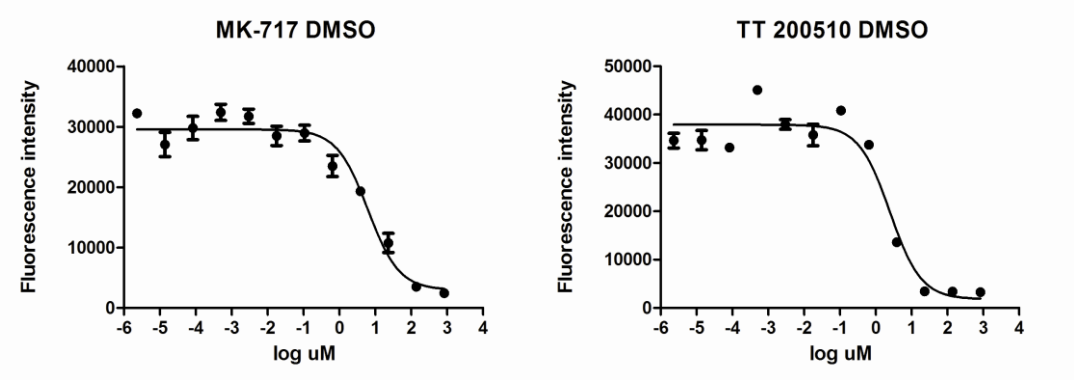


Figure 16: Fluorescence vs. log of concentration of the respective ANP in DMSO

Table 3: Summary of detected EC50 values:

EC50	H_2O as solvent	DMSO as a solvent
MK-717	82μM	6μM
TT-200510	3μM	2.5 μM

4.3. HGXPRT genes in T. brucei

In the *T. brucei* genome there are three genes annotated as HGXPRTs.

```
I               MHSGHPLKPNFVGRDADGNVTVDGRSYPMAESVVATESTIHRSMKEMAQTLANAYKTLKH
II              ------MEP--------------ACKYDFATSVLFTEAELHTRMRGVAQRIADDYSNCNL
III             ------MEP--------------ACKYDFATSVLFTEAELHTRMRGVAQRIADDYSNCNL
                      ::*              . .* :* **: **: :*  *: :** :*: *.. :

I               RDTHNKGNSALAPITDENPLIIISVLKGSYIFTADMVRYLGDCGLPNVVDFIRITSYRGT
II              KPLEN-------------PLVIVSVLKGSFVFTADMVRILGDFGVPTRVEFLRASSYGHD
III             KPLEN-------------PLVVVSVLRGSFVFTADMVRILGDFGVPTRVEFLRASSYGHD
                :  .*             **:::***:**::******* *** *:*. *:*:* :**

I               TKSSGTVQVLDNLRFTELTGKHVLIMEDIADTGRTMKLLVEKIRREYRPASLKVCVLVDK
II              TKSCGRVDVKADG-LCDIRGKHVLVLEDILDTALTLREVVDSLKKSE-PASIKT-LAIDK
III             TKSCGRVDVKADG-LCDIRGKHVLVLEDILDTALTLREVVDSLKKSE-PASIKT-LAIDK
                ***.* *:*  :  : :: *****::*** **. *:: :*:.:::.  ***:*. : :**

I               PGGRVVDFKPEFVCLTAPTRYVVGYGFEVNDRYRNYRHVFVLKPEYAKRYPSKL------
II              PGGRKIPFTAEYVVADVPNVFVVGYGLDYDQSYREVRDVVILKPSVYETWGKELERRKAA
III             PGGRKIPFTAEYVVADVPNVFVVGYGLDYDQSYREVRDVVILKPSVYETWGKELERRKAA
                **** : *..*:*   .*. :*****:: :: **: *.*.:***.  : : .:*

I               -----
II              GEAKR
III             VKAKL
```

I – Tb927.10.1390

II – Tb927.10.1400

III – Tb927.10.1470

All three genes encode for the enzyme HGXPRT, showing only slight differences in their sequences. As can be seen in the alignment of Tb1390, Tb1400 and Tb1470, the latter two are almost identical, with the exception of last 5 amino acids. The Tb1400 and Tb1470 are also very similar to each other at the nucleotide level. This similarity allowed us to target both genes (Tb1400 and Tb1470) using one RNAi construct.

4.4. Cloning into p2T7-177 vector

The desired genes had to be cloned into the RNAi vector p2T7-177 (Wickstead *et al*., 2002).Both genes (Tb927.101390 and Tb927.10.1400 (Tb1390 and Tb1400, respectively) were amplified using specific primers by PCR. produced and the amounts amplified by PCR.

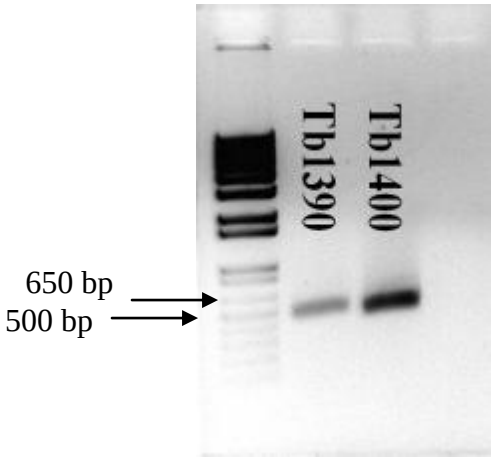


Figure 17: PCR of Tb1390 and Tb1400. Arrows depict the position of expected products. Left lane – 1Kb marker. PCR revealed one specific band corresponding to Tb1390 and Tb1400 at an approximate length of 600 base pairs. This size is consistent with the expected size.

The DNA was purified from the gel and DNA concentration was determined:

Tb1390 14.9 ng/µL

Tb1400 31 ng/µL

The PCR product was ligated into pGEM-T easy, transformed into *E. coli* XL-1 blue cells and two white colonies for each construct were chosen for further analysis. The plasmid DNA was purified from a bacterial culture, digested with appropriate restriction enzymes and the reactions were run on an agarose gel. As obvious from Figure 18, the plasmids Tb1390/1, Tb1400/1 and Tb1400/2 carried the correct insert. Tb1390/2 plasmid seemed to be empty.

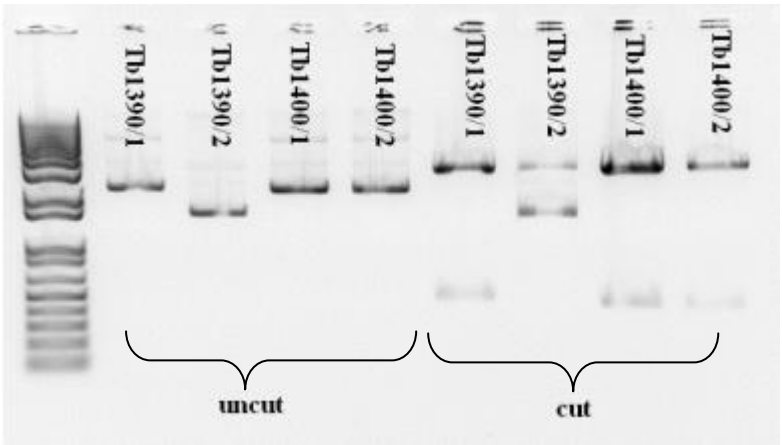


Figure 18: Restriction analysis of the purified plasmids pGEM Tb1390/1 and 2, pGEM Tb1400/1 and 2. The DNA marker is depicted on the left: In this gel, two samples of each plasmid purification were loaded, one cut and one uncut.

The plasmid DNA Tb1390/1 and Tb1400/1 were sequenced to verify correctness of the cloning. Further, the Tb1390 and Tb1400 inserts (cut with XhoI and HindIII) were sub-cloned into p2T7-177 vector (also cut with XhoI and HindIII enzymes). The bacterial colonies were screened using PCR or restriction. The chosen clones p2T7 177 Tb1400/7 and p2T7 177 Tb1390/3 were verified by restriction reaction (Figure 19) and sequenced.

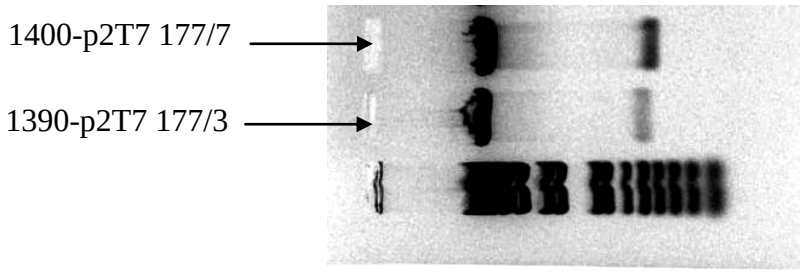


Figure 19: Restriction digest of two selected plasmid DNA p2T7 177 Tb1400/7 and p2T7 177 Tb1390/3.

These two plasmid DNA were then purified in high amounts (Tb1390 2.416µg/µl, Tb1400 1.991µg/µl), digested with NotI enzyme and the linearized DNA (Figure 20) was electroporated into the SM BF *T. brucei* cells.

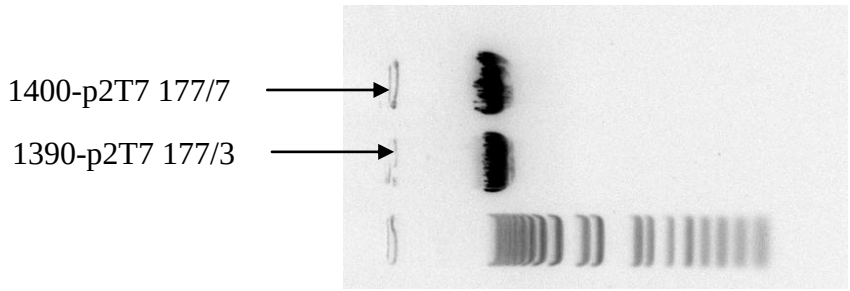


Figure 20: Restriction digest of two selected plasmid DNA p2T7 177 Tb1400/7 and p2T7 177 Tb1390/3

4.5. Growth curves

Six cell lines (three for each construct) were selected for further experiment. First, the stabilates were prepared for each cell line in order to keep the cells in liquid nitrogen. Second, to evaluate if Tb1390 and Tb1400 are essential for the survival of BF *T. brucei* parasites the cells were divided into two groups: non-induced cells and RNAi induced cells. Both types of cells were split down daily to $1x10^5$ cells/ml and the RNAi induced cells were kept under tetracycline induction (1µg/ml). The cells were counted and split every day for 8 days.

The Tb1400 RNAi cell lines did not show any growth phenotype (Fig. 21).

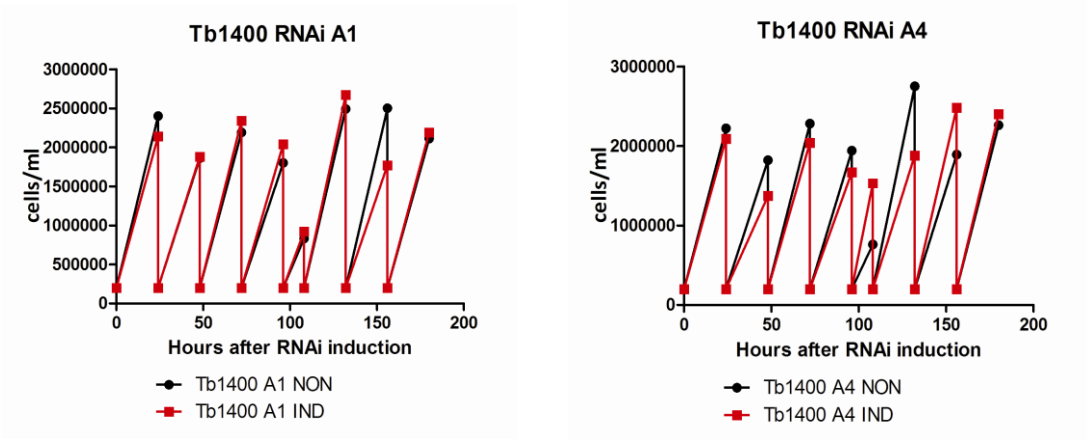


Figure 21. Growth curves of the non-induced (NON) and tet induced (IND) Tb1400 RNAi bloodstream *T. brucei* cell lines A1 and A4. Cells were split every day to maintain their exponential growth phase (between 10^5 – 10^6 cells/ml).

On the other hand, cell lines in which expression of Tb1390 was silenced showed a slight growth inhibition by day 2 and 3 after RNAi induction (Fig. 22).

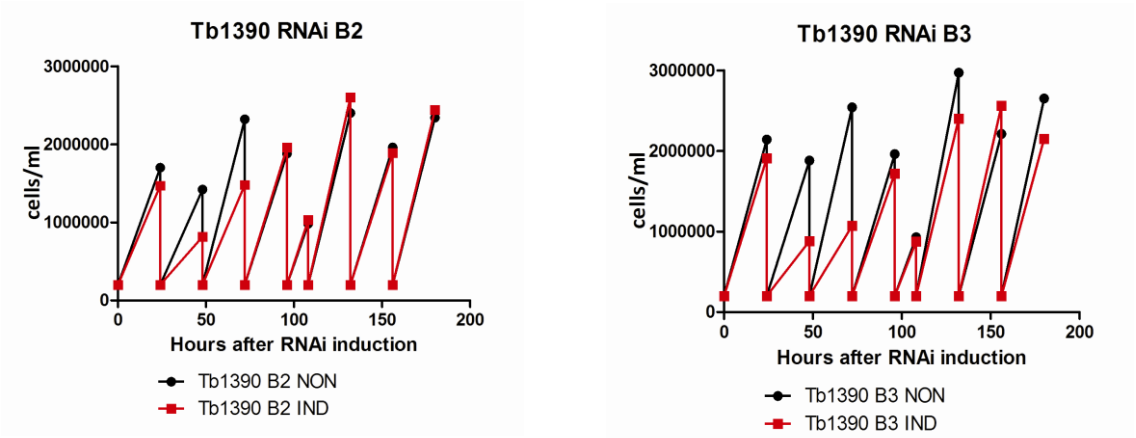


Figure 22. Growth curves of the non-induced (NON) and tet induced (IND) Tb1390 RNAi bloodstream *T. brucei* cell lines B2 and B3. Cells were split every day to maintain their exponential growth phase (between 10^5 – 10^6 cells/ml).

This slight growth phenotype in the case of Tb1390 RNAi might be explained by a different affinity of Tb1390 and Tb1400 to purine nucleotides available in the HMI-9 medium. These two enzymes function in a same mode; however their affinity to distinct nucleotides might be different. Thus the cells might have to adjust their purine metabolism to actual conditions.

5. Conclusions and future perspectives

5.1. Compound screening

Out of 16 ANPs, only two compounds showed a significant inhibition of the BF *T. brucei.* Both drugs are lipophilic ester prodrugs of free ANPs, which may better penetrate through the plasma membrane. TT 200510 had a cytotoxic effect at $2.5\mu M$ concentration while MK 717 showed a significant effect only when dissolved in DMSO. Since these drugs are lipophilic, the solubility is a difficult issue which might be solved by synthesizing different types of prodrugs with improved solubility. MK 717 contains adenine as a purine molecule, while TT 200510 contains guanine. Thus, MK 717 may inhibit TbAPRT enzyme responsible for converting adenine to AMP. On the other hand, TT 200510 most likely binds to Tb 6-oxo purine PRTs. It is noteworthy to mention that the most potent ANPs is S-HPMPA (Kaminski *et al*., 1996), indicating that TbAPRTs are essential for the proper function of *T.brucei* purine salvage pathway. .

In other studies which involved testing several ANPs as inhibitors of *Plasmodium falciparum* HGPRT, Keough *et al*. demonstrated that both the purine base as well as the phosphonate moiety has significant influence on the affinity of the ANPs towards the active site of the enzyme. They were able to show that in comparison to the other purine bases, the ANPs with guanine presented with the highest affinity to the binding site of HGPRT, leading to better inhibition. Their screening also revealed the best pick for the phosphonate moiety, which appears to be a 2-(phosphonoethoxy)ethyl group.

From the analysis of crystal structures of human HGPRT-ANP complexes, the authors deduced that the location of the phosphonate group in relation to the purine base is essential for tight binding. The 5'-phosphate binding site exhibits the same amino acid residues in human HGPRT as in *P. falciparum* HGXPRT, so it can be safely concluded that the finds are relevant for the parasitic enzyme as well. At the phosphate binding site, the phosphonate group is held in place by hydrogen bonds, which only allow for binding in close proximity, limiting the possibilities. This observation might possibly explain why, for example, the compound TT 200510 showed more effect than the very similar compound TT 280510, as the position of the phosphonate group in the later molecule is tightly bound in a ring and positioned closer to the purine base. Another feature of a similar structure in human HGPRT and *P. falciparum* HGXPRT is a mobile loop (64% amino acid sequence identity), which becomes ordered and closed over the active site in the presence of a transition state analogue.

From crystal structure analysis of the HGPRT-ANP complexes, it was shown that when (S)-HPMPG binds, this loop moves, resulting in the formation of a hydrogen bond to one of the oxygen atoms of the phosphonate group (Keough *et al*.; 2009). This explains at least in part the tight binding of the HPMPG and similar phosphonate moieties. Not all the factors and influences on the binding strength of certain phosphonate groups or purine bases are well understood or known and the activity of the two promising compounds from this screening of ANPs are still weak in comparison to the cytotoxicity of pentamidine towards *T. brucei* cells. The groundwork has been laid by the crystal structure analysis of Keough *et al*. for the design and discovery of new drugs, by contributing to the understanding of how these compounds bind in the enzyme HGPRT.

5.2. RNAi

The single knock-down of the two HGXPRT genes in *T.brucei* did not show any or only slight growth phenotype, indicating that the parasite can bypass the lack of at least one of the two enzymes. This observation is in agreement with similar studies done in *Leishmania donovani* (Boitz, Ullman; 2006). *L. donovani* is the causative agent for visceral Leishmaniasis, an often fatal disease in humans, with only toxic and partially ineffective treatment available. The genera of *Leishmania* and *Trypanosoma* are alike in many respects, one of them being the purine salvage pathway. *L. donovani* lacks the *de novo* pathway for purines and relies on its purine salvage enzymes including HGPRT, XPRT, APRT and AK to supply the necessary nutrients (Allen *et al*.; 2005; Jardim *et al*.; 1999; Sinha *et al*.; 1999). As in Trypanosomes, the purine salvage pathway includes several alternative, redundant pathways which can bypass a non-functional enzyme, complicating the identification of valid drug targets. In a study on the purine salvage pathway in *L.donovani*, Boitz and Ullman successfully created and characterized a mutant strain with double-knock down of HGPRT and XPRT. The ability of the mutant strain to proliferate in several different purine sources was assessed, to determine its viability without either of the two enzymes. In previous studies, it had already been confirmed that none of the enzymes was essential by itself (Jardim *et al.;* 1999; Sinta *et al.;* 1999; Hwang *et al*.; 1996; Iovannisci, Ullman, 1984). It could be shown that the double-knock down strain of *L.donovani* could only maintain continuous growth in media with adenosine or adenine. Only a small number of the mutant cells were observed by the end of the experiment, but they were not able to further proliferate and died soon after the nutrient source had been withdrawn (Boitz, Ullman; 2006).

As the mutant parasite was unable to grow in any other media or sustain an infection in mouse macrophages, it was demonstrated that the enzymes HGPRT and XPRT are absolutely essential for purine acquisition, parasite viability and parasite infectivity (Boitz, Ullman; 2006). This find could be exploited by introducing a strategy of live vaccination against Leishmaniasis. A strain with intrinsic mutations could be used to provoke a protective immune response, this has already proven possibly with susceptible rodents (Breton *et al*.; 2995; Uzonna *et al*.; 2004). To sustain the infection, dietary supplements would provide the necessary adenosine until the immune response has been established and the parasites flushed out when the nutrients are withdrawn (Boitz, Ullman; 2006). It remains to be seen if a similar approach might be feasible for the treatment of Trypanosomiasis. Importantly, the double RNAi knock-down of Tb1390 and Tb1400 showed that if the expression of both enzymes is silenced these proteins are essential for BF *T. brucei* survival (Kotrbová and Ziková, Validation of acyclic nucleoside phosphanates as inhibitors of 6-oxo purine phosphorybosyltransferases in *Trypanosoma brucei*. BSc. Thesis). The next steps will be to decipher affinity of Tb1390 and Tb1400 to certain 6-oxo purines using recombinant Tb1390 and Tb1400 proteins. Further *in vivo* studies using more ANP compounds and genetically modified *T. brucei* cell lines (Tb APRT, XPRT a HGPRT RNAi cells, cells over expressing these enzymes etc.) will help to elucidate if purine salvage pathway is a good drug target and if ANPs are promising drug leads.

6. References

Allen T, Hwang HY, Wilson K, Hanson S, Jardim A, and Ullman B. Cloning and expression of the adenine phosphoribosyltransferase gene from Leishmania donovani.Mol Biochem Parasitol. 1995 Oct;74(1):99-103.

Allen TE, Hwang HY, Jardim A, Olafson R, Ullman B.Cloning and expression of the hypoxanthine-guanine phosphoribosyltransferase from Leishmania donovani.Mol Biochem Parasitol. 1995 Jul;73(1-2):133-43.

Barrett MP.The rise and fall of sleeping sickness.Lancet. 2006 Apr 29;367(9520):1377-8.

Berg M, Van der Veken P, Goeminne A, Haemers A, Augustyns K. Inhibitors of the purine salvage pathway: a valuable approach for antiprotozoal chemotherapy? Curr Med Chem. 2010;17(23):2456-81.

Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, Böhme U, Hannick L, Aslett MA, Shallom J, Marcello L, Hou L, Wickstead B, Alsmark UC, Arrowsmith C, Atkin RJ, Barron AJ, Bringaud F, Brooks K, Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivens A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Larke N, Landfear S, Larkin C, Leech V, Line A, Lord A, Macleod A, Mooney PJ, Moule S, Martin DM, Morgan GW, Mungall K, Norbertczak H, Ormond D, Pai G, Peacock CS, Peterson J, Quail MA, Rabbinowitsch E, Rajandream MA, Reitter C, Salzberg SL, Sanders M, Schobel S, Sharp S, Simmonds M, Simpson AJ, Tallon L, Turner CM, Tait A, Tivey AR, Van Aken S, Walker D, Wanless D, Wang S, White B, White O, Whitehead S, Woodward J, Wortman J, Adams MD, Embley TM, Gull K, Ullu E, Barry JD, Fairlamb AH, Opperdoes F, Barrell BG, Donelson JE, Hall N, Fraser CM, Melville SE, El-Sayed NM.The genome of the African trypanosome Trypanosoma brucei.Science. 2005 Jul 15;309(5733):416-22.

Boitz JM, Ullman B. A Conditional Mutant Deficient in Hypoxanthine-guanine Phosphoribosyltransferase and Xanthine Phosphoribosyltransferase Validates the Purine Salvage Pathway of Leishmania donovani.J Biol Chem. 2006 Jun 9;281(23):16084-9. Epub 2006 Apr 8.

Breton M, Tremblay MJ, Ouellette M, Papadopoulou B. Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis.Infect Immun.2005 Oct;73(10):6372-82.

Busby S., Ebright RH. Transcription activation by catabolite activator protein (CAP). J. Mol. Biol. 2001;293: 199–213.

Hansen LH, Knudsen S, Sørensen SJ. The effect of the lacY gene on the induction of IPTG inducible promoters, studied in Escherichia coli and Pseudomonas fluorescens. Curr. Microbiol. 1998 Jun;36(6):341-7.

Hwang, H. Y., Gilberts, T., Jardim, A., Shih, S., and Ullman, B. (1996) J. Biol. Chem. 271, 30840–30846

Iovannisci DM, and Ullman B.Characterization of a mutant Leishmania donovani deficient in adenosine kinase activity. Mol Biochem Parasitol. 1984 Jun;12(2):139-51.

Jabs CM, Neglen P, Eklof B, Thomas EJ. Adenosine, inosine, and hypoxanthine/xanthine measured in tissue and plasma by a luminescence method. Clin Chem. 1990 Jan;36(1):81-7.

Jardim A, Bergeson SE, Shih S, Carter N, Lucas RW, Merlin G, Myler PJ, Stuart K, Ullman B.Xanthine phosphoribosyltransferase from Leishmania donovani. Molecular cloning, biochemical characterization, and genetic analysis.J Biol Chem.1999Nov26;274(48):34403-10.

Keough DT, Brereton IM, de Jersey J, Guddat LW.The crystal structure of free human hypoxanthine-guanine phosphoribosyltransferase reveals extensive conformational plasticity throughout the catalytic cycle.J Mol Biol. 2005 Aug 5;351(1):170-81.

Keough DT, Hocková D, Holý A, Naesens LM, Skinner-Adams TS, Jersey J, Guddat LW.Inhibition of hypoxanthine-guanine phosphoribosyltransferase by acyclic nucleoside phosphonates: a new class of antimalarial therapeutics.J Med Chem.2009Jul23;52(14):4391-9.

Monzani PS, Trapani S, Thiemann OH, Oliva G. Crystal structure of Leishmania tarentolae hypoxanthine-guanine phosphoribosyltransferase. BMC Struct Biol. 2007 Sep 25;7:59.

Ngô H, Tschudi C, Gull K, Ullu E. Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. Proc Natl Acad Sci U S A. 1998 Dec 8;95(25):14687-92.

Opperdoes FR, Borst P . Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome. FEBS Lett. 1977 Aug 15;80(2):360-4.

Peacock L, Ferris V, Bailey M, Gibson W.Fly transmission and mating of Trypanosoma brucei brucei strain 427.Mol Biochem Parasitol. 2008 Aug;160(2):100-6. Epub 2008 Apr 30.

Sinha KM, Ghosh M, Das I, Datta AK. Molecular cloning and expression of adenosine kinase from Leishmania donovani: identification of unconventional P-loop motif.

Biochem J. 1999 May 1;339 (Pt 3):667-73.

Räz B, Iten M, Grether-Bühler Y, Kaminsky R, Brun R. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (T.b. rhodesiense and T.b. gambiense) in vitro. Acta Trop. 1997 Nov;68(2):139-47.

Slowiaczek P, Tattersall MH. The determination of purine levels in human and mouse plasma. Anal Biochem. 1982 Sep 1;125(1):6-12. No abstract available.

Ullman B, Carter D. Molecular and biochemical studies on the hypoxanthine-guanine phosphoribosyltransferases of the pathogenic haemoflagellates. Int J Parasitol. 1997 Feb;27(2):203-13.

Uzonna JE, Späth GF, Beverley SM, Scott P.Vaccination with phosphoglycan-deficient Leishmania major protects highly susceptible mice from virulent challenge without inducing a strong Th1 response.J Immunol. 2004 Mar 15;172(6):3793-7.

Vanhamme L, Paturiaux-Hanocq F, Poelvoorde P, *et al.* . Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature. March 2003.422 (6927): 83–7.

Wickstead B, Ersfeld K, Gull K. Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of Trypanosoma brucei. Mol Biochem Parasitol. 2002 Nov-Dec;125(1-2):211-6.

Wirtz E, Leal S, Ochatt C, Cross GA. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in Trypanosoma brucei, Mol Biochem Parasitol. 1999 Mar 15;99(1):89-101.

Internet sources:

University College London: www.ucl.ac.uk (accessed April 28th, 2012)
MIT Open Course Ware: ocw.mit.edu/index.htm, (April 28th, 2012)
www.agscientific.com/ (accessed April 28th, 2012)
www.di.uq.edu.au/proj4aproc (accessed March 22nd 2012)
WHO: www.who.int/mediacentre/factsheets/fs259/en/ (accessed Dec 28th 2011)